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ORIGINAL ARTICLE

Assessment of immunological anti-platelet factor 4 antibodies for vaccine-induced thrombotic thrombocytopenia (VITT) in a large Australian cohort: A multicenter study comprising 1284 patients

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Abstract

Background: Vaccine-induced thrombotic thrombocytopenia (VITT) is a rare complication of adenovirus-based vaccines aimed to prevent and minimize COVID-19 and related pathophysiology.

Objectives: To describe patterns of testing for anti-platelet factor 4 (PF4) antibodies using various ELISA assays in a large Australian cohort and comparative functional platelet activation assays in a subset.

Patients/Methods: Asserachrom HPIA IgG ELISA was performed in 1284 patients over a period of 12 months, supplemented in select cohorts by comparative ELISA using three other methods ($n = 78–179$), three different functional assays (flow cytometry, serotonin release assay, and/or Multiplate; $n = 476$), and rapid immunological chemiluminescence anti-PF4 assay ($n = 460$), in a multicenter study.

Results: For first episode presentations, 190/1284 (14.8%) ELISA tests were positive. Conversely, most (445/460; 96.7%) chemiluminescence anti-PF4 test results were negative. All functional assays showed associations of higher median ELISA optical density with functional positivity and with high rates of ELISA positivity (64.0% to 85.2%). Data also identified functional positivity in 14.8%–36.0% of ELISA negative samples, suggesting false negative VITT by HPIA IgG ELISA in upward of one third of assessable cases.

Conclusion: To our knowledge, this is the largest multicenter evaluation of anti-PF4 testing for investigation of VITT. Discrepancies in test results (ELISA vs. ELISA or ELISA vs. functional assay) in some patients highlighted limitations in relying on single methods (ELISA and functional) for PF4 antibody detection in VITT, and also highlights the variability in phenotypic test presentation and pathomechanism of VITT.

KEYWORDS
COVID-19 vaccine, enzyme-linked immunosorbent assay, platelet factor 4, thrombocytopenia, thrombosis

1 | INTRODUCTION

Vaccine-induced (immune) thrombotic thrombocytopenia (VITT) is a rare complication of adenovirus-based vaccines against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which are aimed to prevent and minimize coronavirus disease 2019 (COVID-19) and related pathophysiology.[1,2] Alternatively called thrombocytopenia with thrombosis syndrome (TTS) by government reporting agencies, such as the Therapeutic Goods Administration (TGA) in Australia, VITT affects 1 in 50 000 to 100 000 individuals vaccinated with adenovirus-based vaccines.[1] VITT mimics heparin-induced thrombocytopenia with thrombosis (HIT or HITT), and reflects generation of platelet activating antibodies directed to complexed platelet factor 4 (PF4).[1–4] In HIT, the antibodies are directed against PF4 complexed to heparin, whereas different PF4 complexes form in VITT.[1–5] Early case series of VITT formed the basis upon which other laboratories began to investigate this immune-mediated disorder.[6–8]

Although many diagnostic guidelines have been published,[9,10] VITT is clinically suspected when a patient develops thrombosis with associated suggestive laboratory findings, following recent exposure to an adenovirus vaccine. Two of the well-established early laboratory signs of potential VITT are thrombocytopenia (or a sharp drop in platelet count; similar to HIT), and highly elevated D-dimer.[3,9–11] However, as VITT is mostly identified in the community (versus HIT being identified in the hospital setting), a sharp

Essentials

- Vaccine-induced thrombotic thrombocytopenia (VITT) is a rare complication of COVID-19 vaccination, with presence of anti–platelet factor 4 antibodies.
- Multicenter antibody testing (ELISA, functional assays) in a large ($n = 1284$) Australian cohort.
- Less than one fifth (14.8%) showed positive ELISA, with functional positivity in one third of ELISA negative samples.
- We highlight limitations of single methods, and variability in test phenotypic presentation and pathomechanism of VITT.

drop in platelet count is less readily detected, and so recognition of thrombocytopenia is relied upon. However, as with HIT, thrombocytopenia may not always be evident in VITT.[12] For example, an early study reported on 11 VITT patients with a normal platelet count, 9 of which later became thrombocytopenic.[12] Confirmation of VITT can be achieved by assessment of anti-PF4 antibodies and functional platelet activation.[1–4,6–9,13] For immunological detection of VITT-associated anti-PF4 antibodies, only ELISA have been shown to be sensitive, while rapid PF4 assays (e.g., lateral flow and chemiluminescent immunoassays) are generally insensitive, contrasting what occurs with HIT.[1–4,6–9,13,14] Moreover, different ELISA assays may vary in sensitivity to these antibodies.[3,4,15] Functional platelet activation tests include modified (e.g., PF4 enhanced) serotonin release assays (SRA) and flow cytometry-based assays; however, unlike HIT, for which low dose heparin is used to augment detection of activating antibodies, heparin may instead reduce *in vitro* platelet activation in functional VITT assays.[3,4] Thus, detection of VITT may require additional modifications to functional assays to make them more sensitive.

Several reports have now been published on the detection of PF4 antibodies in VITT (recently reviewed in Favaloro[3] and Favaloro et al.[4]). Most have been case studies or small case series. We herein describe a multicenter investigation of immunologically detectable anti-PF4 antibodies in an Australian cohort comprising 1284 patients referred for VITT-specific testing, with all laboratories harmonizing to use the same commercial ELISA kit. Of importance, Australia was heavily reliant on one adenovirus-based vaccine (AstraZeneca ChAdOx1-nCoV-19/Vaxzevria) for a long period of the initial COVID-19 vaccination drive, with 13.8 million doses administered in Australia up to May 19, 2022.[16] However, alternativee vaccines became recommended in those under 60 years of age following reports of Australian cases of thrombosis with TTS by the TGA, and since the end of 2021, very few doses of adenovirus-based vaccine are now being used.[16] Nevertheless, adenovirus-based vaccines continue to be employed throughout the world. To our knowledge, this is the largest number of cases investigated for VITT by immunological evaluation of PF4 antibodies in a single study.

2 | MATERIALS AND METHODS

2.1 | Overview of setting, study design, and timeline considerations

This evaluation was undertaken by clinicians and scientific staff at several Australian hospital sites in a co-ordinated Australia-wide approach to investigation of VITT (Figure S1 in supporting information). The first VITT case series was pre-published on March 28, 2021,[17] and formed the basis of a later peer-review publication.[6]

Under the auspices of the Thrombosis and Haemostasis society of Australia and New Zealand (THANZ), the Australasian (Australia and New Zealand) VITT Advisory Group was established on March 26, 2021, with the first VITT Advisory Statement published online on the THANZ website (https://www.thanz.org.au/) on April 1, 2021. A secondary group of hospital scientists and hematologists was formed to undertake co-ordinated testing for PF4 antibodies (VITT ELISA Test Group), with the first ELISA assay performed on a subsequently confirmed VITT patient on April 1, 2021.[18] Over the subsequent 12 months, the group has performed ELISA testing on 1284 patients being investigated for VITT, with this comprising a total of 1476 ELISA assays using a single manufacturer product (see Section 2.3). Testing was performed in six different public hospital-affiliated centers in five states of Australia (Figure S2 in supporting information). No testing was performed in New Zealand, and there were no cases of VITT identified to our knowledge in New Zealand (the AstraZeneca vaccine was never deployed there). ELISA testing was managed by each site, with local hematologist oversight to triage samples based on clinical probability for VITT, to first determine which patient samples were tested by ELISA, and then if considered necessary, referral for additional functional testing. The situation in the state of New South Wales (NSW) differed in that local ELISA testing was performed at two separate sites, with oversight, co-ordination, and sample triaging being primarily managed centrally by the Concord team, first for ELISA testing, and then if deemed appropriate, for functional platelet activation testing, for both local NSW and nationally submitted samples. In context, the group was constantly cognizant of resourcing issues (testing during a period of significant disruption of global supply chains; staff availability; PF4 ELISA kit availability and possible future supply issues; and assay throughput limitations, especially for functional testing); thus, not all samples were assessed in all assays. Capacity limitations drove some decisions regarding differential functional testing. Most testing, ELISA and functional, was performed within diagnostic centers, otherwise busy with other diagnostic testing, and without additional deployment of resources. Throughput limitations were especially evident for functional assays, for which maximum capacity runs tended to be in single digit patient numbers. The process is further explained below and in Figure S2 and Figure 1.

2.2 | Pre-ELISA/functional test probability assessment for VITT

To enable laboratories to manage the expected imminent burden of VITT-related testing, the Australian VITT advisory group formulated a pre-test probability assessment for VITT (Table S1 in supporting information).[19,20] Thus, patients were characterized as "probable," "possible," "less likely," and "much less likely" for VITT, on the basis of platelet count/thrombocytopenia, level of D-dimer (and/or if available fibrinogen), and evidence of thrombosis, with timing relative to receiving ChAd-Ox1 vaccination appropriate for potential antibody induction (4–42 days post dose as the final temporal criterion). Based on these criteria, samples were triaged and progressed to ELISA testing (or not), and furthermore, progressed

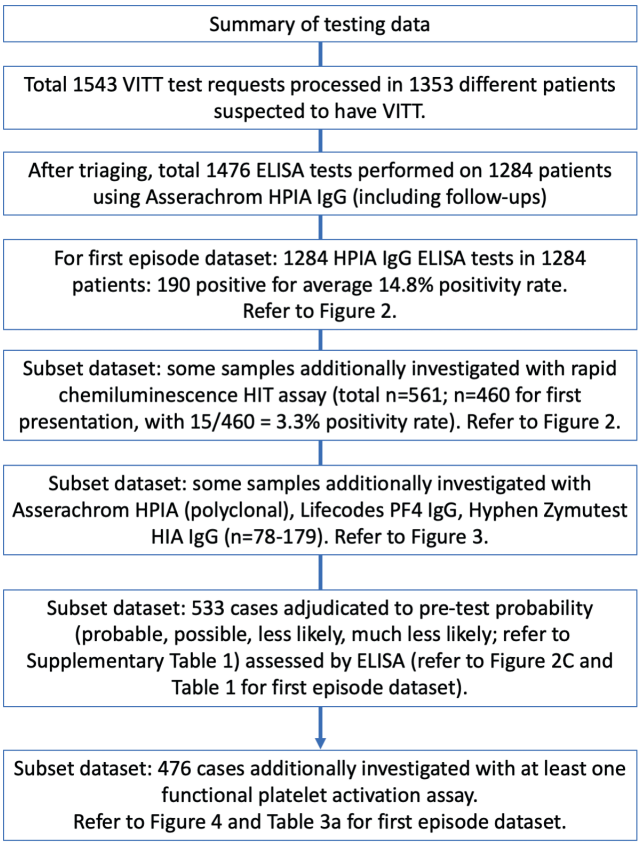


FIGURE 1 Study flow chart showing summary of testing data. HIT, heparin-induced thrombocytopenia; VITT, vaccine-induced thrombotic thrombocytopenia.

to functional testing (or not; Figure S2). Some patients could not be assigned to a category due to missing information.

2.3 | ELISA testing for VITT

Due to the requirement for immediate establishment of a diagnostic pathway for VITT or its exclusion, the VITT Advisory Group decided to progress VITT ELISA testing re-purposing the most prevalent pre-established method for HIT ELISA in Australia, the Asserachrom HPIA IgG (Cat. No. 00624; Stago Diagnostics) assay.[14] This decision was influenced by limited local availability of alternative ELISA assays, which were also not easily or immediately accessible in Australia, with implementation of alternative ELISA platforms also potentially challenged by lack of local accreditation, delays from extra staff training, and local assay validation. Instead, local verification data, at least for HIT investigations, was available for the Asserachrom HPIA IgG assay.[14] Moreover, data using the Asserachrom HPIA IgG ELISA assay quickly emerged in the international literature, and was identified in several studies to be appropriately sensitive to VITT.[3,4,15] As the assessment of VITT progressed, it however also became clear that not all patients clinically strongly suspected to have VITT were positive with this assay, and thus select patients were also assessed using a variety of other ELISA assays, including Asserachrom HPIA (Cat. No. 00615; Stago Diagnostics) polyclonal assay (anti-heparin/PF4 IgA, IgG, and IgM antibodies), Lifecodes PF4 IgG (Immucor; Product code HAT45G; purchased from Immulab), and Zymutest HIA IgG (HYPHEN Biomed; Cat. No. RK040A; from Haematex; Figure 1). All testing was performed according to manufacturer's instructions, and tests were interpreted as positive or negative for anti-PF4 antibodies using the manufacturer's cut-offs, albeit these were originally derived for the diagnosis of HIT.

2.4 | Rapid anti-PF4 immunological testing

Although not expected to be positive in VITT,[3,4,15] limited testing was undertaken at some sites using rapid anti-PF4 testing using a variety of non–ELISA based assays, but primarily AcuStar HIT-IgG (PF4-H) (Chemiluminescence method, Cat. No. 009802032; ACL AcuStar). In part, this was to provide potential data for differential identification of VITT versus HIT (or even "spontaneous HIT-like syndrome"), because it was not always clear if a patient being investigated for VITT had recent proximal heparin therapy or other precipitating event.[21] All testing was performed according to manufacturer guidance for HIT testing, as no alternative instructions for VITT testing were available.

2.5 | Functional testing for PF4-related platelet activation

The Concord team was responsible for managing and triaging functional testing for platelet activation. Detailed findings with functional testing are the subject of separate publications in progress, but we established three different approaches to functional testing at three different centers. This was required because of capacity limits at each site performing functional assays, such that no single laboratory could provide functional testing of all samples that were deemed to need functional testing. These tests comprised SRA,[14] a recently described procoagulant platelet flow cytometry assay,[22] and multiplate multiple electrode testing. Methods were based on HIT-related assays,[14,23–27] with some modifications to improve sensitivity for VITT (vs. HIT) introduced over time, including assessments in the absence of heparin (all assays), effect of potential inclusion of purified human PF4 on platelet activation (flow and SRA assays), and specificity with assessment of effect of low (therapeutic) and high (supra-therapeutic) dose heparin (all assays) and Fcgamma receptor blocking antibody, IV.3, on platelet activation (flow assay). Thus, each patient investigation comprised at least six different treatments assessed by flow,[22] three to five different conditions assessed by SRA, and three conditions assessed by multiplate (manuscripts under preparation). In distinction from public health definitions of TTS, functional testing was considered essential for a clinical diagnosis of "pathological" VITT, similar to use of functional testing to confirm "pathological" HIT.[14,25,26] Due to resourcing constraints (staff availability, test availability, etc.), functional testing was primarily

performed on patients that fit a "probable" or "possible" pre-test limit probability for VITT (Table S1), although for quality assurance purposes occasional samples from other classification groups were also tested to provide true negative cases.[22]

2.6 | Ethical considerations

For the flow cytometry evaluations, human studies were approved by Sydney Local Health District Human Research Ethics Committees (HREC/18/CRGH/294, X21-0160, 2021/ETH00945). Healthy donors gave written informed consent. For the remaining studies, based on guidance from local human research ethics committees, additional and separate ethical approval for the diagnostic study was not required, as the evaluation represented a Quality Assurance project of method verification for use in diagnostics.

3 | RESULTS

3.1 | Summary data

In total, 1543 patient samples were referred for VITT testing, from 1353 different patients (Figure 1). A total of 1476 ELISA tests were performed in 1284 patients using Stago Asserachrom HPIA IgG over 12 months (Figure 1). Restricting samples to first patient presentation episode, a total of 1284 patients had ELISA assays performed, with 190 positive (14.8%; Figure 1). Similar positivity rates were observed at all test sites, with similar median optical density (OD) values (Figure S3A in supporting information). A subset of patients ($n = 460$) had rapid anti-PF4 testing by chemiluminescence assay, with 15/460 (3.3%) positive.

A summary of categorical OD data for the HPIA IgG ELISA for first episode data is shown in Figure 2A, alongside data using the rapid chemiluminescence-based AcuStar HIT assay. Across participating sites, negative Asserachrom HPIA IgG ELISA ODs tended to be under 0.3 OD units, while positive ODs ranged from 0.15 to above 3.0; thus, ODs between 0.15 and 0.3 could be identified as negative or positive depending on the cut-off on day of testing, but ODs above 0.3 were invariably identified as positive. The results of ELISA testing of the same sample on different ELISA test runs, either in the same lab or in a different lab is shown in Figure S3B. There was some variation in OD values, but in general, the classification as negative or positive remained unchanged. As expected, most (445/460; 96.7%) first episode AcuStar anti-PF4 test results were negative; however, 15 patients yielded positive findings. Most AcuStar positive samples were also ELISA positive (14/15, 93.3%; Figure 2B).

3.2 | ELISA data according to pre-test probability assessment

Data for Asserachrom HPIA IgG ELISA results according to pre-test VITT probability for the first episode dataset is shown in Figure 2C, with further detail provided in Table 1. A total of 167 cases were classified as "probable VITT" (/1284 patents = 13.0%). The median OD was substantially higher in the "probable" group than in the other groups (Figure 2C) and there were a substantially higher proportion of positive ELISA results in the "probable" group, followed sequentially by the "possible," "less likely," and "much less likely" groups (Table 1). As noted in Table 1, not all cases could be assigned to a pre-test group due to missing clinical information. The ELISA ODs obtained by these non-assigned cases is shown in Figure S3C. Most cases were ELISA negative. Comparable data for the fourT score–derived pre-test probability for HIT versus positivity for the same ELISA assay using a historical cohort[14] assessed for HIT is shown in Figure 2D.

3.3 | Comparative ELISA data using additional ELISA methods

Given the possibility of false negative ELISA data using the Asserachrom HPIA IgG ELISA, select patient samples were also co-assessed using a variety of other ELISA methods by several sites, primarily as a quality control assessment, to evaluate variation in results, and as a possible contingent in the event of disruption to supply of the Asserachrom assay. These ELISA-compared patient samples included several unexpected Asserachrom HPIA IgG negative samples from patients in the VITT probable group, and several unexpected Asserachrom HPIA IgG positive samples from patients in the VITT "less likely/much less likely" groups. Unsurprisingly, there was variation in findings according to ELISA assay (Figure 3). Although there was mostly concordance of positivity or negativity with most samples across ELISA assays, some patient samples were positive with one assay but negative by another (Figure 3A, B, C). There was a sufficient number of data points to undertake a sub-analysis of the Asserachrom HPIA IgG versus Hyphen Zymutest HIA IgG according to pre-ELISA test probability assessment, as well as the post-test adjudication (see Table S1). The probable VITT group yielded a higher median OD for the Hyphen Zymutest HIA IgG assay than for the Stago HPIA IgG (Figure 3D), as well as a higher number of positive cases (Table 2), indicating that in this selected cohort, some of the Stago HPIA IgG negative cases were Hyphen HIA IgG positive. There was also better separation of data with the Hyphen HIA IgG for "probable" compared to the combined "less likely and much less likely group" (Figure 3D, Table 2). Finally, the comparative data for Asserachrom HPIA IgG versus Hyphen HIA IgG for VITT adjudicated cases is shown in Figure 3E. Again, the Hyphen HIA IgG assay yielded higher median ODs for co-tested samples when cases were adjudicated to be serologically confirmed.

3.4 | ELISA data compared to functional platelet activation assays

The relationship between Asserachrom HPIA IgG ELISA OD and SRA, flow assay, and multiplate assay, is shown in Figure 4, as well

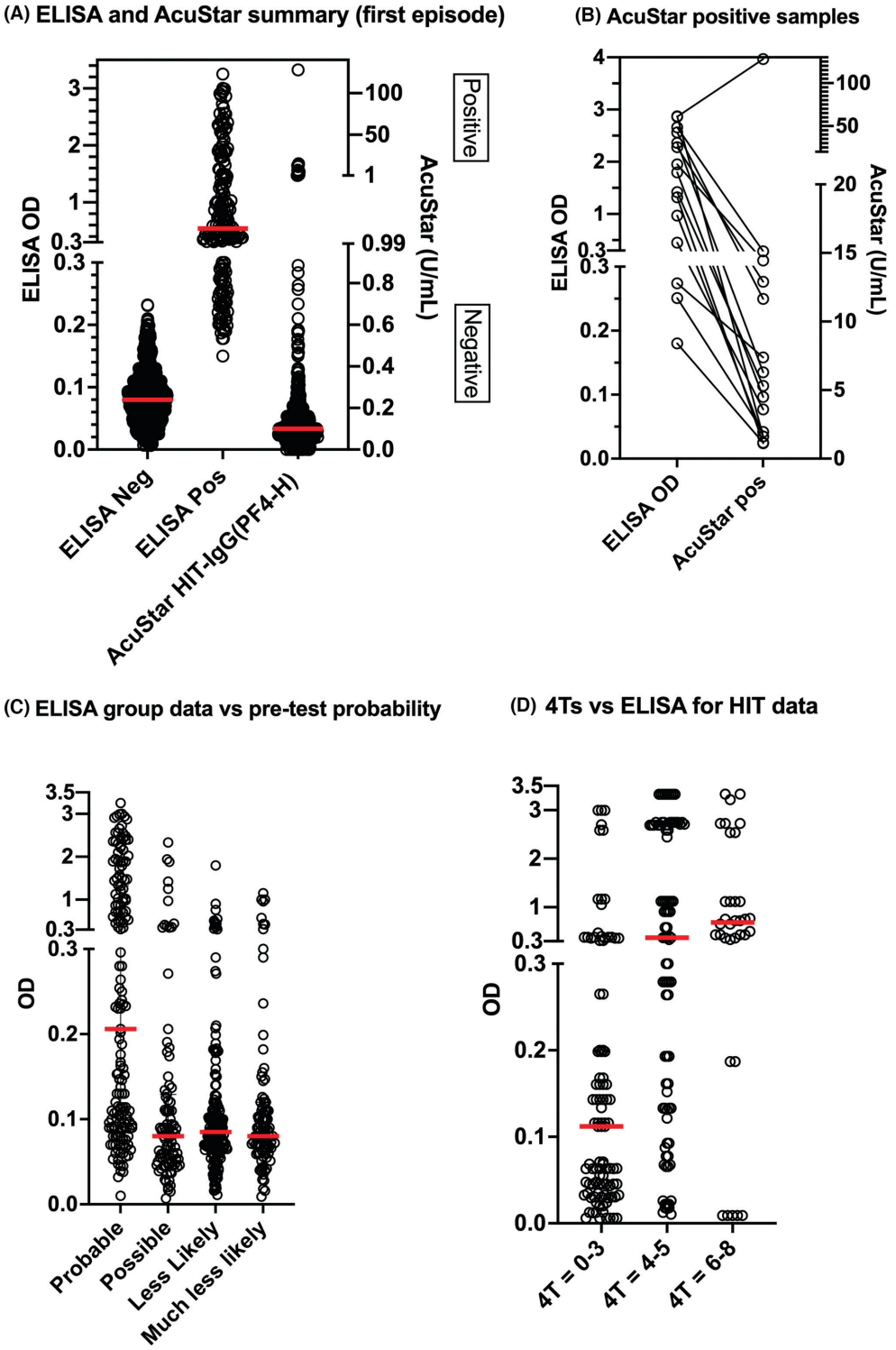


FIGURE 2 Some summary data for first episode immunological testing of anti–platelet factor 4 (PF4) antibodies in the Australian cohort. A, Entirety of first episode ELISA data for Asserachrom HPIA IgG ($n = 1284$) separated into negative ($n = 1094$) and positive ($n = 190$), with comparative data using the chemiluminescence based assay on AcuStar ($n = 490$). Samples defined as ELISA negative had optical density (OD) readings under 0.3. In total, 14.8% of ELISA assays were reported as positive. In contrast, only 3.3% (15/490) AcuStar assays were reported as positive (i.e., ≥1.0 U/mL). B, AcuStar positive samples ($n = 15$) were mostly (14/15; 93.3%) also Asserachrom HPIA IgG ELISA positive. C, Asserachrom HPIA IgG ELISA ODs versus vaccine-induced thrombotic thrombocytopenia (VITT) pre-ELISA test probability grading. The VITT "probable" group had higher median OD, and also showed proportionally higher numbers of ELISA positives (numbers as per Table 1). D, Historical cohort[14] of patients evaluated for heparin-induced thrombocytopenia (HIT), with Asserachrom HPIA IgG ELISA ODs plotted according to 4Ts grade (low = 0–3 [$n = 100$]; intermediate = 4–5 [$n = 89$]; high = 6–8 [$n = 34$]).

TABLE 1 Number of cases assigned to different pre-ELISA test VITT probability groups, as well as proportional positive ELISA rate[a]

	Probable	Possible	Less likely group	Much less likely group	Alternative reason for thrombocytopenia/thrombosis
n	167	93	183	90	70
ELISA pos (n)	85	15	21	10	4
ELISA neg (n)	82	78	162	80	66
% ELISA pos	**50.9**	**16.1**	**11.5**	**11.1**	**5.7**

Abbreviation: VITT, vaccine-induced thrombotic thrombocytopenia.

[a]Data for Stago Asserachrom HPIA IgG ELISA for first episode cases. Not all cases could be assigned to a group due to missing clinical and/or laboratory data. Classification for "probable," "possible," "less likely," and "much less likely" groups as per Table S1 in supporting information. The "alternate reason for thrombocytopenia/thrombosis" group comprises vaccine, thrombocytopenia, thrombosis, and raised D-dimer, but alternative cause for presentation identified. This last group yielded a % ELISA positive value close to the expected background rate of ELISA positives post vaccination unrelated to VITT. The chi-square statistic is 91.7443. The *p*-value is <.00001. The result is significant at $p < .05$.

as summarized in Table 3 for the final adjudicated VITT cases. All functional assays showed an association of higher median ELISA OD with functional positivity (Figure 4A, B, C, D, E, F, G). Moreover, positivity in each functional assay was associated with a high rate of ELISA assay positivity, ranging from 64.0% to 85.2% (Table 3). Similarly, negativity in functional assays was associated with a high rate of ELISA assay negativity, ranging from 65.1% to 82.1% (Table 3). However, data also showed functional positivity in 14.8%–36.0% of ELISA negative samples, which might represent false negative VITT by Asserachrom HPIA IgG ELISA in upward of one third of assessable (Table 3A) and clinically adjudicated (Table 3B) cases.

Figure 4 also shows data for flow and SRA assays separated into groups with or without added PF4. The modification using added PF4 aimed to increase functional assay sensitivity, but overall data with versus without added PF4 was similar in the assessed samples. Moreover, some individual cases tested with both approaches were positive with added PF4 and negative without, and vice versa. The overall percentage of cases adjudicated to be "VITT-serologically confirmed" and positive with each different functional method is shown in Figure 4H.

3.5 | Other data

Figure 5 shows summary data for the additional items of days to onset (presentation post vaccination; Figure 5A), presentation platelet count (Figure 5B), and D-dimer levels (Figure 5C), for various patient categories post adjudication.

4 | DISCUSSION

To our knowledge, this is the largest multicenter evaluation of anti-PF4 testing for investigation of VITT. There are some notable findings that we can highlight. First, proportional detection of ELISA positive cases was similar across all ELISA test sites, with 14.8% overall positivity for first episode cases. That most patient samples investigated for VITT were identified to be negative for anti-PF4 antibodies is unsurprising, and akin to findings with HIT.[14]

A potential distinction, however, is that while experience with HIT evaluations covers decades of research work, the timeline for VITT evaluation comprises only 1 year. For the VITT investigated cases, we could identify some likely ELISA false negatives and also some likely ELISA false positives. It needs to be recognized that a background rate of anti-PF4 antibodies (approximately 5%) can be observed in normal individuals post vaccination, with these antibodies detected by ELISA, but not causing platelet activation.[28,29] However, as there is no gold standard test for VITT, it is not always clear whether a false negative or false positive ELISA has occurred. Some clues may arise by evaluating different ELISA assay results. Thus, high OD ELISA values, especially if seen with two different ELISA assays, likely reflect true positives, especially if confirmed by a functional assay. Indeed, we felt that functional confirmation of pathological VITT, analogous to functional confirmation of pathological HIT, was critical to diagnosis of VITT, and functional positivity was seen in a substantive portion of ELISA negative cases. Because gold standards are missing, we cannot exclude the possibility of false positive functional assays. However, as we applied a triaging process in which functional testing was primarily performed in patients with high VITT probability, we believe the risk of false positive functional assays is low. Functionally positive cases may include a subset of non-PF4 dependent cases. Thus, we would conclude that isolated ELISA testing without functional testing has limited scope for diagnosis of VITT, as there is a risk of false negatives using a single ELISA platform. This may differ to experience with HIT testing, in which ELISA assays are known to overcall the possibility of HIT, and thus give rise to a high rate (up to 50%) of false positives.[14]

Alternatively, low OD values identified as negative by two different ELISA assays likely reflect true negatives, but discrepant results warrant further testing, especially those in the "probable VITT" group. Our data suggest, but cannot confirm, that the Hyphen Zymutest HIA IgG likely represents a more discriminative ELISA assay for VITT than the Stago Asserachrom HPIA IgG ELISA, as shown by the clearer association with pre-ELISA test probability assessment and reflected by higher rates of positive results in the probable and VITT serologically confirmed groups (Figure 3D,E and Table 2). However, as these were selected samples, we cannot exclude the possibility of selection bias.

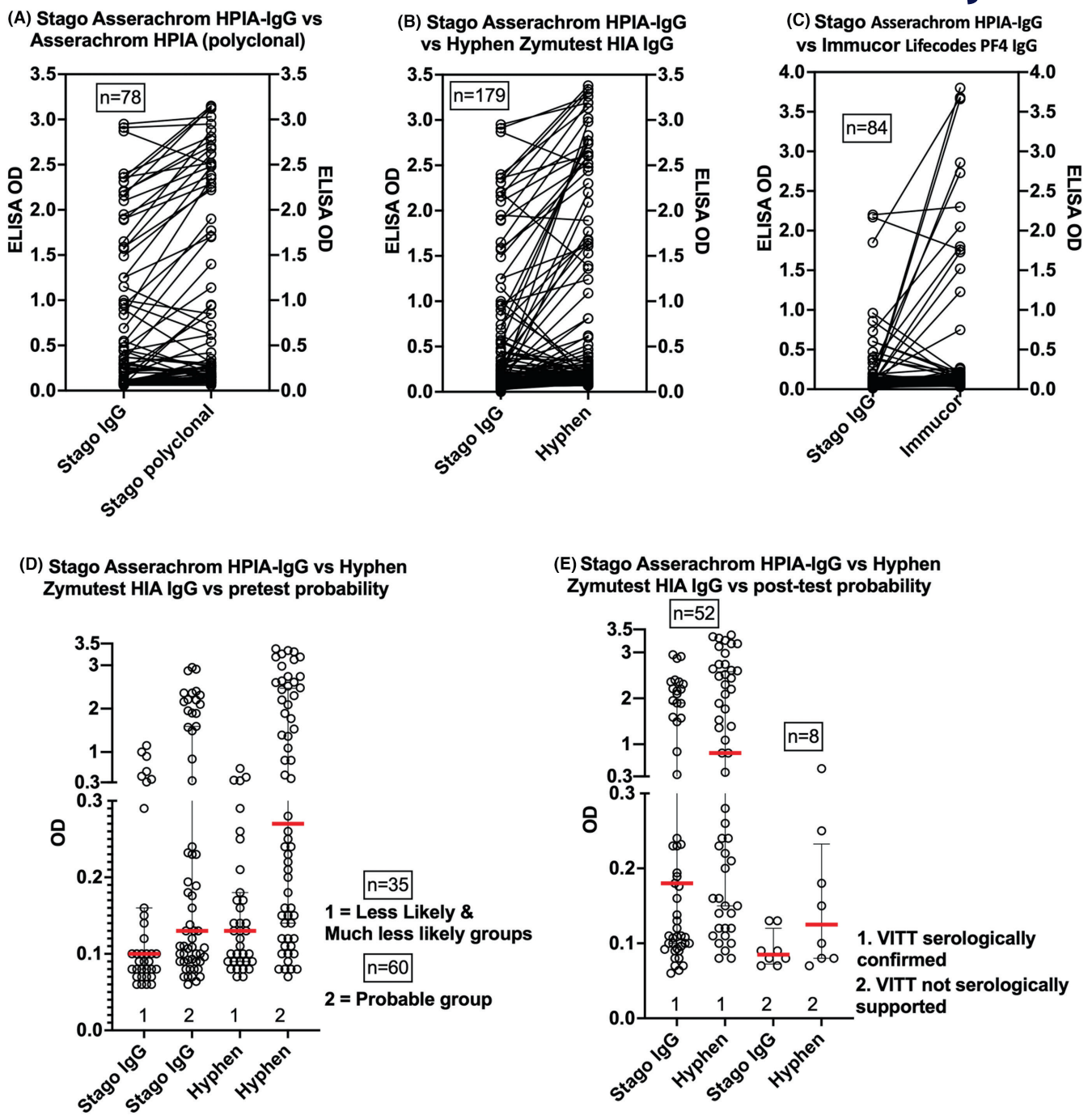


FIGURE 3 Comparison of Asserachrom HPIA IgG ELISA optical density (OD) data versus select data using alternative anti–platelet factor 4 (PF4) ELISA assays. Numbers of samples co-assessed in each comparison is shown in each figure. Data include some samples selected because they showed unexpectedly negative Asserachrom HPIA IgG ELISA results in patients identified to have "probable" vaccine-induced thrombotic thrombocytopenia (VITT), and unexpectedly positive Asserachrom HPIA IgG ELISA results in patients identified to have "less likely and much less likely" VITT. Most positive and negative Asserachrom HPIA IgG ELISA results were also respectively positive and negative with other ELISA assays; however, some samples were positive with one assay, but negative with another. A, Stago Asserachrom HPIA IgG ELISA versus Stago Asserachrom HPIA (polyclonal) ELISA. B, Stago Asserachrom HPIA IgG ELISA versus Hyphen Zymutest HIA IgG ELISA. C, Stago Asserachrom HPIA IgG ELISA versus Immucor anti-PF4 IgG ELISA. D, Comparison of Stago Asserachrom HPIA IgG ELISA versus Hyphen Zymutest HIA IgG ELISA in terms of VITT probability "grade" (i.e., "less likely/much less likely" groups combined vs. "probable"). For this select data group, the Hyphen assay seemed to show better separation of positive versus negative ELISAs according to VITT probability "grade" (see also Table 2). E, Comparison of Stago Asserachrom HPIA IgG ELISA versus Hyphen Zymutest HIA IgG ELISA in terms of VITT serological confirmation. For this select data group, the Hyphen assay also seemed to show better separation of positive versus negative ELISAs according to VITT serological confirmation.

TABLE 2 Number of cases assigned to different pre-ELISA test VITT probability groups, as well as proportional positive ELISA rate for different ELISA assays[a]

	STAGO Asserachrom HPIA IgG		Hyphen Zymutest HIA IgG	
	Probable group	Less likely & much less likely groups	Probable group	Less likely & much less likely groups
n	60	35	60	35
Pos (*n*)	22	8	29	3
Neg (*n*)	38	27	31	32
% Pos	**36.7**	**22.9**	**48.3**	**8.6**

Abbreviation: VITT, vaccine-induced thrombotic thrombocytopenia.

[a]Select cases tested for both Asserachrom HPIA IgG and Zymutest HIA IgG ELISA assays as per Figure 3D. Classification for "probable" and "less likely and much less likely" groups as per Table S1 in supporting information. There were insufficient patients with a "possible" classification for inclusion here.

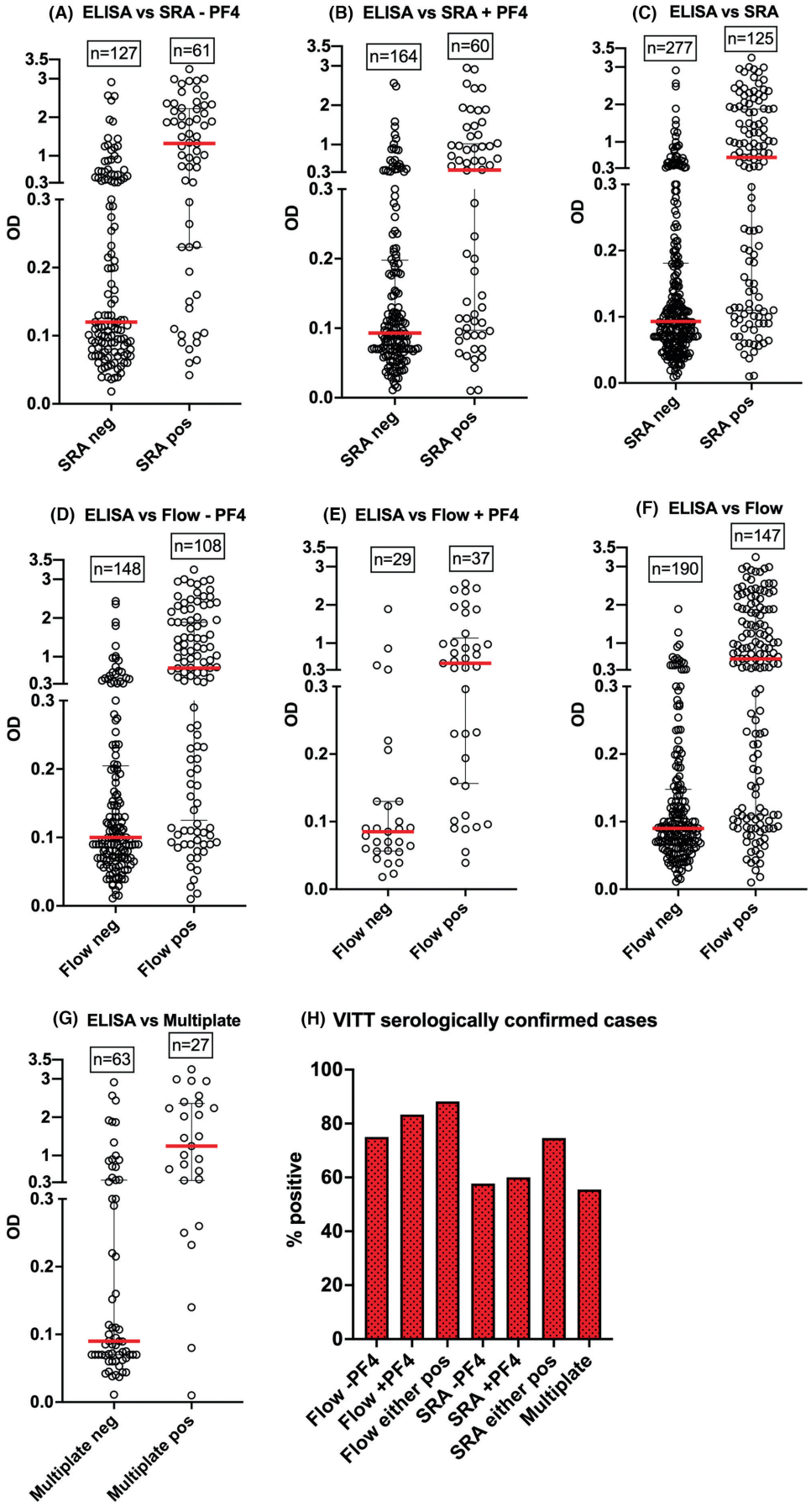
We also saw variability in functional test results, with some cases positive with one assay, but negative by another. Interestingly, as a group, there did not appear to be a substantive difference in functional flow or SRA whether or not PF4 was added to the assay (Figure 4). However, on a case-by-case basis, some cases were positive by PF4 supplemented assays and negative without, and vice versa. This suggests that VITT is more heterogenous than previously appreciated, and likely more heterogeneous than HIT. In terms of functional assays, SRA and flow-based assays appeared to better identify platelet activation than multiplate analysis (Figure 4H).

ELISA data have been previously presented in many case studies and small case series (reviewed in references[3,4]). Naturally, such reporting is influenced by case ascertainment bias. Platton et al.,[15] in an early UK study reported on 43 cases being investigated for VITT, with 27 "probable" VITT based on criteria similar to ours and assessed ELISA results with four different ELISA assays. The three ELISA systems evaluated in our study showed similar sensitivity to VITT in the Platton et al. study,[15] with medians of 91%–94%; interestingly, Asserachrom HPIA IgG ELISA was reported to have the highest specificity for VITT (up to 100%). The same panel of four ELISA assays was also evaluated in an external quality assessment exercise comprising one case of VITT, one case of HIT, and one non-HIT/VITT sample as sent to 85 laboratories.[30] Another cross-laboratory evaluation sent sera from 12 well-defined VITT patients to four laboratories using five different PF4/heparin immunoassays.[31] These assays thus used prescreened samples to conclude that these could be detected in most laboratories with most assays. Also, a combination of a positive ELISA and a negative AcuStar CLIA might be useful to identify VITT antibodies in the absence of confirmatory functional assays.

A recent case series from British Columbia reported on 68 patients investigated for VITT, with only three confirmed to have positive ELISA and confirmatory SRA.[32] Another recent publication from Germany reported on post vaccination adverse events in 854 cases of thrombosis, and 224 cases of thrombocytopenia, in which 69 cases of VITT were suspected, 52 of which had a positive PF4 ELISA and functional test.[33]

We acknowledge some strengths and also some limitations in our study. We investigated a large cohort ($n = 1284$) of patients with one ELISA assay with good concordance of results across multiple sites. This reflects a high level of harmonization in approach, probably underpinned by experience in HIT ELISA testing, staff training, and quality control processes, and this permitted the development of a truly national testing program with extensive rural and regional coverage.[19] This in turn led to early identification, improved management, and we believe reduced morbidity and mortality in the Australian cohort. The TGA of Australia reported a total of 8 deaths in Australia from a total of 172 cases of TTS (i.e., 4.7% case fatality rate), from about 13.8 million total doses of AstraZeneca vaccine administered in Australia.[16] This compares very well from descriptions of 30%–40% mortality in early VITT cohorts.[3] We developed predefined data collection items, referral patterns, standardized case definitions (although these evolved over time), permitting early standardization, facilitating subsequent refinements, and allowing centralized triaging and adjudication. Another strength was the use of three separate functional assays to help adjudicate VITT confirmation, and to supplement ELISA testing. Although this was done primarily to share the burden of testing across three sites, comparative data also permits us to draw some conclusions regarding potential relative utility. In terms of limitations, we acknowledge the lack of any clear gold standard with which to clearly categorize cases; however, this is not new in diagnostic tests for new or evolving diseases. Another weakness is the variability in OD readings on a day-to-day basis, limiting utility of OD cut-offs or patient readings per se, and which may be a target for future improvement.

FIGURE 4 Data for Stago Asserachrom HPIA IgG ELISA optical densities (ODs) versus functional platelet activation assays separated according to functional assay positivity. Numbers of samples assessed in each comparison is shown in each figure. Functional assay positivity was associated with higher (but similar) median ELISA ODs for all assays, as well as higher proportion of ELISA positivity (Table 3). Stago Asserachrom HPIA IgG ELISA versus: (A) SRA (no added platelet factor 4 [PF4]); (B) SRA (with added PF4); (C) SRA (irrespective of added PF4; positive group = positive with added PF4 and/or without); (D) flow assay (no added PF4); (E) flow assay (with added PF4); (F) flow assay (irrespective of added PF4; positive group = positive with added PF4 and/or without); (G) multiplate assay. (H) Percentage positivity rate for each functional assay for the final adjudicated "VITT [vaccine-induced thrombotic thrombocytopenia] serologically confirmed" cohort.


(A) ELISA vs SRA - PF4
n=127
n=61
OD
SRA neg
SRA pos
(B) ELISA vs SRA + PF4
n=164
n=60
OD
SRA neg
SRA pos
(C) ELISA vs SRA
n=277
n=125
OD
SRA neg
SRA pos
(D) ELISA vs Flow - PF4
n=148
n=108
OD
Flow neg
Flow pos
(E) ELISA vs Flow + PF4
n=29
n=37
OD
Flow neg
Flow pos
(F) ELISA vs Flow
n=190
n=147
OD
Flow neg
Flow pos
(G) ELISA vs Multiplate
n=63
n=27
OD
Multiplate neg
Multiplate pos
(H) VITT serologically confirmed cases
% positive
Flow -PF4
Flow +PF4
Flow either pos
SRA -PF4
SRA +PF4
SRA either pos
Multiplate

5 | CONCLUSIONS

The early establishment of the VITT advisory group facilitated a truly national harmonized approach to investigation of VITT, and a low overall case mortality applying TTS data from the TGA.[16] The ELISA group collaborated to manage reagent use, facilitating ongoing uninterrupted testing for the patient cohort with the highest probability of VITT. Up to one third of ELISA negative patients with clinical criteria for VITT were functional assay positive. The possibility of relatively high rates of ELISA false negatives, along with variation in the test patterns for functional assays, such that some cases were positive by one assay, but negative by another, or variably positive with added PF4 or not, suggests that VITT

TABLE 3 Relationship between ELISA positivity[a] and functional platelet activation assays

	Flow positive	Flow negative	SRA positive	SRA negative	Multiplate positive	Multiplate negative
(A) All cases tested						
ELISA (*n*)	147	190	125	277	27	63
ELISA pos (*n*)	98	34	80	65	23	22
ELISA neg (*n*)	49	156	45	212	4	41
% ELISA pos	**66.7**	17.9	**64.0**	23.5	**85.2**	34.9
% ELISA neg	33.3	**82.1**	36.0	**76.5**	14.8	**65.1**
(B) Adjudicated cases only (VITT serologically confirmed)						
ELISA (*n*)	84	13	83	26	16	14
ELISA pos (*n*)	58	6	64	12	14	9
ELISA neg (*n*)	26	7	19	14	2	5
% ELISA pos	**69.0**	46.2	**77.1**	46.2	**87.5**	**64.3**
% ELISA neg	31.0	**53.8**	22.9	**53.8**	12.5	35.7

Abbreviation: VITT, vaccine-induced thrombotic thrombocytopenia.

[a]Using Stago Asserachrom HPIA IgG ELISA.

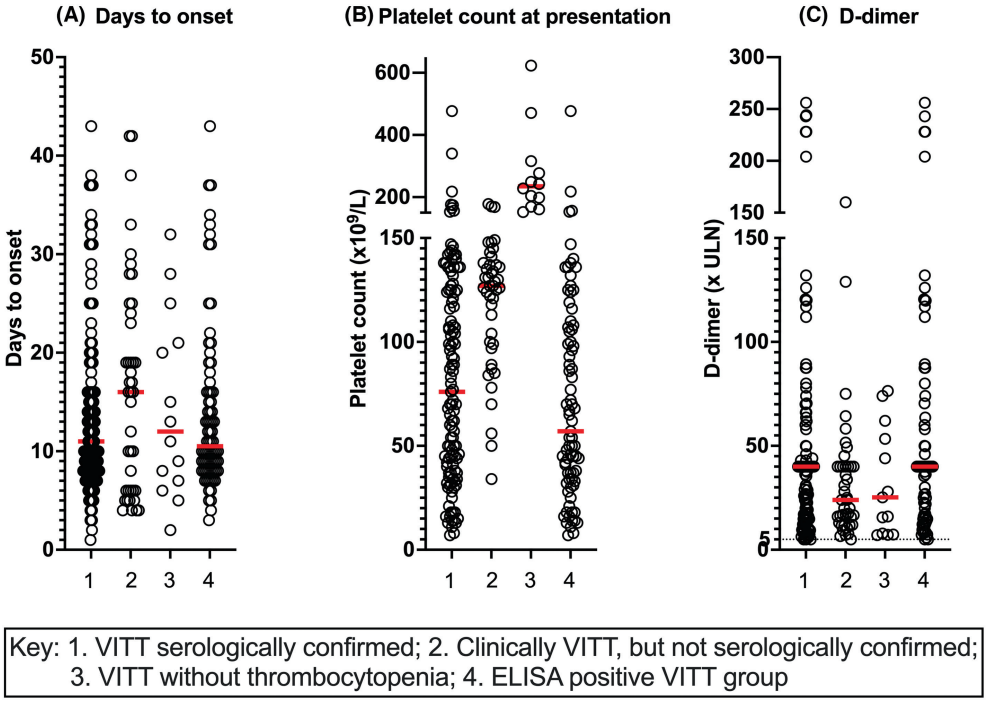


FIGURE 5 Additional data for other items: (A) days to onset (presentation post vaccination); (B) presentation platelet count, and (C) presentation D-dimer levels (x upper limit of normal [ULN]), for various patient categories post adjudication (i.e., vaccine-induced thrombotic thrombocytopenia [VITT] serologically confirmed group; group adjudicated to be clinically VITT, but not serologically confirmed; VITT group without thrombocytopenia; VITT serologically confirmed group restricted to ELISA positive patients only).

may be more heterogeneous than HIT. Discordance between ELISA platforms suggests that in the absence of functional testing, two different ELISA assays may be required to more fully capture VITT cases.

AUTHOR CONTRIBUTIONS

All authors contributed to the study concept and design, and/or to the acquisition of clinical and/or experimental data. VMC acted as the lead for the VITT Advisory Group, and EJF acted as the lead for the ELISA group. The Concord group (primarily VMC, LC, and CL) undertook case evaluation in order to triage cases for ELISA testing and functional testing performed in NSW. At other state centers, the hematologists were responsible for clinical review of cases to assess the need for ELISA testing, and the scientists responsible for ELISA based testing at that center. A core group of the authors (VMC, TB, LC, FP, HT, SC) were responsible for post-test adjudication for VITT. EJF undertook the bulk of the final data and statistical analysis, wrote the original draft manuscript, and prepared the figures. All authors contributed to critical revision of the manuscript and provided their final approval for submission.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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